Inducible Metabolic Adaptation Promotes Mesenchymal Stem Cell Therapy for Ischemia
A Hypoxia-Induced and Glycogen-Based Energy Prestorage Strategy
Hongming Zhu, Aijun Sun, Yunzeng Zou, Junbo Ge

Objective—Ischemic tissue is an environment with limited oxygen and nutrition availability. The poor retention of mesenchymal stem cells (MSC) in ischemic tissues greatly limits their therapeutic potential. The aim of this study was to determine whether and how inducible metabolic adaptation enhances MSC survival and therapy under ischemia.

Approach and Results—MSC were subjected to glycogen synthase 1–specific small interfering RNA or vehicle treatment, and then sublethal hypoxic preconditioning (HP) was applied to induce glycogenesis. The treated cells were subjected to ischemic challenge. The results exhibited that HP of MSC induced glycogen storage and stimulated glycogen catabolism and cellular ATP production, thereby preserving cell viability in long-term ischemia. In vivo study using the mouse limb ischemia model transplanted with HP or control MSC into the ischemic thigh muscles revealed a significant increased retention of MSC with glycogen storage associated with improved limb salvage, perfusion recovery and angiogenesis in the ischemic muscles. In contrast, glycogen synthesis inhibition significantly abolished these improvements. Further molecular analysis indicated that phosphoinositide 3-kinase/AKT, hypoxia-inducible factor-1, and glycogen synthase kinase-3β regulated expression of glycogenesis genes, including glucose transporter 1, hexokinase, phosphoglucomutase 1, glycogen synthase 1, and glycogen phosphorylase, thereby regulating glycogen metabolism of stem cell during HP.

Conclusions—HP-induced glycogen storage improves MSC survival and therapy in ischemic tissues. Thus, inducible metabolic adaptation in stem cells may be considered as a novel strategy for potentiating stem cell therapy for ischemia. (Arterioscler Thromb Vasc Biol. 2014;34:870-876.)

Key Words: energy metabolism ▪ hypoxia ▪ hypoxia-inducible factor 1 ▪ limb ischemia ▪ mesenchymal stromal cells

Mesenchymal stem cells (MSC) are multipotent stromal cells from bone marrow and other adult tissues. MSCs are capable of differentiating into adipogenic, osteogenic, and chondrogenic lineages.1 Transplantation of bone marrow–derived MSC has been considered a promising treatment for ischemic tissue injuries, such as myocardial infarction and hindlimb ischemia.2, 3 Nonetheless, the poor retention of implanted MSC in injury site greatly limits the therapeutic potential of MSC.3−5

It is well-known that stem cells negotiate their surrounding nutritional and signaling environments and respond accordingly in functions and fates.10 Under hypoxic/ischemic environment, oxygen-independent glycolysis becomes the critical means for cells to generate ATP. To maintain this anaerobic metabolism for long-term survival of MSC after severe and continuous hypoxia, persistent glucose supply is required.11, 12 Glycogen is a polymer of glucose residues in cytoplasm.13 Glycogen plays an important role in glucose metabolism and forms an energy reserve that can be quickly mobilized to meet the sudden need for energy.14−16 Here, we used sublethal hypoxic preconditioning (HP), a promising tool for clinical practice,17, 18 to preactivate cellular glycogenesis. We test the hypothesis that inducible glycogen storage appeases energy shortage of MSC in ischemia, thereby promoting survival of MSC and their therapeutic efficiency. Our results showed that HP regulated glycogen metabolism of MSC through phosphoinositide 3-kinase (PI3K)/AKT and hypoxia-inducible factor-1 (HIF-1)/glycogen synthase kinase-3β (GSK-3β)–mediated pathways, which in turn promoted survival of MSC and their therapeutic efficacy for ischemic injuries.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Result
HP Promoted Glycogenesis in MSC via Glycogen Synthase 1
To test our hypothesis, we first evaluated cellular glycogen storage. Experimental grouping was showed in the Table. As seen in Figure 1A, all groups of MSC displayed detectable
levels of glycogen using periodic acid-schiff staining. The cells with HP show more positive staining than control. Glycogen synthase 1 (GS1) small interfering RNA inhibited HP-induced glycogenesis. These observations were confirmed further by a quantitative analysis (biochemical titration) of the glycogen content (Figure 1B). We defined the initial glycogen amount of MSC as basal line. After incubation for 24 hours in hypoxia, MSC showed an increase in the amount of glycogen (1.81-folds over the basal value). However, such rise was inhibited by GS1 silencing. Taken together, these results indicate that hypoxia stimulates glycogen formation of MSC via GS1. We also performed a time course study to determine the kinetics of glycogen synthesis and degradation under both normoxia and ischemia. In consequence, glycogen was accumulated during HP and was later consumed in ischemia. Meanwhile, the normoxia of MSC did not affect glycogenesis, thus showing low cell viability in long-term ischemia (Figure 1C and 1D).

**HP-Induced Glycogen Storage Promoted MSC Survival and Therapeutic Efficiency in Subsequent Ischemia**

We went on to test whether glycogen storage provides an adaptive response for MSC to encounter the subsequent hypoxic/ischemic microenvironment (Figure 2). A volume of 10^6 MSCs were injected into the ischemic limb of mice, the retention of these cells was determined using in vivo tracking and histological analysis. Hypoxia preconditioning increased the ratio of survival MSC (green fluorescent protein signal), although these increases were significantly attenuated by GS1 silencing (Figure 2A and 2B). These in vivo cellular retention observations were determined using histological analysis (Figure 2C and 2D). The green fluorescent protein signal that displays in brown was stained using the horseradish peroxidase-diaminobenzidine system.

In light of the finding that glycogen storage restored MSC viability and retention, we went on to examine the effect of glycogen storage on the MSC therapeutic efficacy. During the morphological assessment of necrosis limb (Figure 3A and 3B), we found that MSC transplantation attenuated limb necrosis, and the HP-MSC showed a more pronounced reduction in grade 2 necrosis compared with con-MSC. However, GS1 silencing effectively abolished all such improvement (with a 3- and 2-fold increase in grade 2 and grade 3 necrosis, respectively). In Doppler perfusion image assay (Figure 3C and 3D), HP-MSC–induced perfusion recovery was inhibited significantly using GS1 silencing (day 5, 56±11% versus 22±8.8%; day 14, 83.1±7.8% versus 33±10.3%). HP-MSC also displayed a significantly improved capillary density in

ischemic tissues (Figure 3E and 3F). Negative control small interfering RNA (HP-conRNA group) did not show any significant effects. In summary, as a fuel source for energy production, glycogen storage is a key survival element to cope with the hostile microenvironment.

**PI3K/AKT Regulates Glycogen Metabolism of MSC Directly and Indirectly Through HIF-1 and GSK-3β Signaling Cascade**

As one of the main switches for cellular response to extracellular stimulation, PI3K/AKT signal plays a vital role in cell adaptation. We investigated the potential role of PI3K/AKT in glycogen storage using its specific inhibitor LY294002.

Using periodic acid-schiff staining and glycogen determination, large aggregates of glycogen particles were observed in MSC under hypoxic conditions (Figure 4A and 4B). However, inhibition of PI3K/AKT nearly abolished HP-induced glycogenesis in MSCs (Figure 4B). PI3K/AKT signaling directly regulated glycogen phosphorylase activity (Figure 4C). Meanwhile, PI3K/AKT inhibition suppressed activation of HIF-1 and GSK-3β (Figure 4D and 4E), which may further regulate glycogen metabolism of MSC indirectly. As shown in Figure 5A and 5B, HIF-1α silencing markedly inhibited glycogen accumulation (1.75±0.15 μg/μg protein versus 1.05±0.22 ng/μg protein; P<0.05). We further studied the genes which participate in glycogenesis, including glucose transporter 1, hexokinase, phosphoglucomutase 1, and GS1. Evidence from immunofluorescence (IF) indicated that these glycogenesis-related genes were upregulated in HP-MSC in a HIF-1–dependent manner, the effects of which are abolished by HIF-1α small interfering RNA silencing. These findings were confirmed by Western blot and quantitative real-time polymerase chain reaction (Figure 5C–5E). In our Figure IV in the online-only Data Supplement, we show that inhibition of these genes may result in the failure of HP-induced glycogen accumulation. Furthermore, HP treatment induced glycogen synthesis at least in part through inhibition of GSK-3β. HP decreased GSK-3β activity and thus stimulated glycogenesis. Upregulation of GSK-3β activity via adenovirus overexpression of GSK-3β significantly inhibited HP-induced glycogenesis (Figure 5F–5H).

**Discussion**

The salient findings from our present study revealed an adaptive role of glycogen prestorage in stem cells under hostile environment, suggesting the benefit of inducible metabolic adaptation strategy in stem cell therapy. Our data further demonstrated that sublethal hypoxia preconditioning activated glycogen-based metabolic adaptation via PI3K/AKT and its downstream signal molecules HIF-1 and GSK-3β to dynamically regulate cellular glycogen storage and degradation in an effort to improve MSC survival and therapy in ischemia.

Despite significant advance during the past decade, massive loss of implanted cell resulted from the posts ischemic hostile microenvironment remains a major obstacle for a meaningful therapeutic remedy. Many strategies have been implemented to overcome such barrier. Unlike gene modification where excessive and continuous expression changes may be detrimental to tissue repair, HP is...
a clinical technique that loads physiologically preconditioned MSC and therefore avoids the unwanted side effects. Previous studies had indicated that HP retards MSC senescence,\(^2^6\) promotes MSC proliferation and migration, as well as elicits paracrine regulation.\(^6,2^7\) However, its effect on stem cell metabolism, especially the metabolic adaptation under the subsequent hostile environment, is largely unknown. Although Estrada et al\(^2^8\) have demonstrated that hypoxic culture activates glycolysis of MSC, several concerns still remain. The glycolysis-based energy production depends on constant glucose supply. Question remains regarding how it works when cells are transplanted into a metabolic material–deficient condition (ischemic tissue) later on? If hypoxia-activated glycolysis is terminated by glucose deficiency in ischemic tissue, how to explain the positive effect of hypoxia on cultured MSC? Is it possible for hypoxia to induce both glycolytic catabolism and energy storage in stem cell at same time? The current study was designed to answer these outstanding questions.

Considering the critical role of glucose supply on ATP production and MSC survival,\(^1^1,2^9\) if one can fulfill the raw material need for energy generation, at least in the acute stage, massive loss of implanted MSC may be prevented. Therefore, we hypothesize that inducible glycogen accumulation may act as the energy storage for MSC metabolic adaptation in ischemia, thereby enhancing MSC therapeutic efficacy. Glycogenesis

![Figure 1](image1.png)

Figure 1. Hypoxic preconditioning (HP) promotes glycogenesis of mesenchymal stem cells (MSCs) via glycogen synthase 1 (GS1). MSCs were subjected to GS1 small interfering RNA (siRNA) or vehicle treatment before HP and glycogen assay. A, Representative images of glycogen staining (using periodic acid-schiff staining). B, Glycogen quantification was analyzed using glycogen titration assay. HP-induced glycogenesis was suppressed greatly by GS1-specific siRNA (n=5). C and D, Time course study. After normoxia or hypoxic treatment, cells were subjected to in vitro ischemic model (1% O\(_2\), in cell culture deprived of serum and glucose). C, Time course response of glycogen content in long-term in vitro ischemia. D, Time course response in cell viability in long-term in vitro ischemia (mean±SEM; *P<0.05 vs con-MSC; #P<0.05 vs HP-MSC).

![Figure 2](image2.png)

Figure 2. Hypoxic preconditioning (HP)–induced glycogen storage promotes mesenchymal stem cells (MSCs) survival in subsequent ischemia. For in vivo experiment, MSCs with different treatments were transplanted into ischemic muscles in mice. Two weeks later, MSCs retention was determined. A and B, Representative figure of in vivo fluorescence imaging and green fluorescent protein (GFP) intensity analysis. The fluorescence signal intensity denotes retention of GFP-labeling MSC in ischemic region. C and D, Representative images and pooled data of histological immunohistochemistry. Implanted MSC was shown in brown color, with nucleus staining in blue (scale bar, 0.1 mm; mean±SEM; n=7; *P<0.05 vs con-MSC; #P<0.05 vs HP-MSC).
can be activated by short hypoxic insult in cancer cells and c2c12 myoblasts.\textsuperscript{14,30} In present study, we demonstrated that cellular glycogen storage was induced in MSC using HP. A previous study\textsuperscript{6} has reported that hypoxia-stimulated glycogen storage is a consequence of the incremented amount of enzyme, rather than its activation change. Therefore, glycogenesis was blocked using GS1-specific small interfering RNA, rather than the short hairpin RNA or enzyme inhibitors.

PI3K/AKT signal is a critical regulator for cellular survival in a wide range of cell types.\textsuperscript{31} Recent studies have identified several substrates for the serine/threonine kinase AKT, suggesting that this kinase blocks cell death though regulation of gene expression involved in cell fate and metabolic flexibility.\textsuperscript{32} HIF-1 and GSK-3\(\beta\) are well-known mediators downstream of AKT for hypoxic response and glycogen metabolism, respectively. In the present study, we found that PI3K/AKT signal regulated glycogen metabolism both directly and indirectly, involving downstream HIF-1 and GSK-3\(\beta\) signaling molecules to regulate glycogenesis-related gene expression. In addition, PI3K/AKT regulated glycogen accumulation through direct regulation of glycogen degradation enzyme (glycogen phosphorylase) activity. It is well

### Table. Experimental Grouping

<table>
<thead>
<tr>
<th>Groups</th>
<th>Con-MSC</th>
<th>HP-MSC</th>
<th>HP-siRNA</th>
<th>HP-conRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxic preconditioning</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Normoxic culture</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Specific siRNA treatment</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Negative control siRNA</td>
<td>–</td>
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<td>–</td>
<td>+</td>
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HP indicates hypoxic preconditioning; MSC, mesenchymal stem cells; and siRNA, small interfering RNA.
perceived that HIF-1 regulates a functional group of genes that associated with glycogen metabolism under hypoxia. Hypoxia promotes glycogen accumulation via HIF-1–induced induction of GS1. Our results further consolidated these observations. Our data showed that the HP activated a series of glycogenesis-related genes, including glucose transporter
Glucose-6-phosphate by hexokinase.

and thus intracellular glucose is rapidly phosphorylated to glycogen. Glucose-6-phosphate is a crucial intermediate for glycogen synthesis and degradation. Phosphoglucomutase 1 (PGM1) is the first enzyme in glycogenesis that catalyzes the conversion of glucose-6-phosphate (G-6-P) into glucose-1-phosphate (G-1-P) and then into glycogen. This enzyme is involved in the regulation of glycogen metabolism through hypoxia-inducible factor-1 (HIF-1) and glycogen phosphorylase (GP) pathway.

During hypoxic preconditioning (HP), MSCs are exposed to hypoxic conditions. This results in the expression of hypoxia-inducible factor-1 (HIF-1), which promotes glycogen accumulation via PI3K/AKT-dependent suppression of glycogen phosphorylase (GP) activity. HIF-1 is a master regulator of glycogen metabolism, and its activation leads to increased glucose uptake, glycogen synthesis, and increased glycogen content in MSCs.

In summary, results from our current study demonstrated that HP turns on glycogen storage and regulates its metabolism. Current data from cell survival and therapeutic functions prove that the glycogen content induced by HP is harmless. However, the current study suffers from several limitations that warrant further investigation: we used hypoxia to trigger glycogen accumulation, which is highly dependent on HIF-1 signal. In addition, many genes involved in glycogen metabolism are downstream of HIF-1. If the glycogen accumulated in normoxia MSC where HIF-1 signal may be suppress. Did it still show enhancing effects on MSC therapy?

In summary, results from our current study demonstrated that HP turns on glycogen storage and regulates its metabolism. This finding implies that HP not only causes glycogen accumulation but also contributes to glycogen degradation and energy transformation when cells were transplanted into ischemia.

Excessive accumulation of glycogen induced by HP may interrupt cellular normal function and promote cellular injury. Current data from cell survival and therapeutic functions prove that the glycogen content induced by HP is harmless. However, the current study suffers from several limitations that warrant further investigation: we used hypoxia to trigger glycogen accumulation, which is highly dependent on HIF-1 signal. In addition, many genes involved in glycogen metabolism are downstream of HIF-1. If the glycogen accumulated in normoxia MSC where HIF-1 signal may be suppress. Did it still show enhancing effects on MSC therapy?

In summary, results from our current study demonstrated that HP turns on glycogen storage and regulates its metabolism via PI3K/AKT and its downstream HIF-1 and GSK-3β signal cascades. Glycogen storage is involved in the MSC metabolic adaptation as energy storage to promote MSC survival and therapeutic efficacy for ischemia. Our findings support a novel metabolic adaptation strategy to improve MSC therapy.

Sources of Funding

This work was supported, in part, by the National Basic Research Program of China (2011CB503905) and Program for New Century Excellent Talents in University.

Disclosures

None.

References


**Significance**

Energy deficiency–induced poor retention of implanted mesenchymal stem cell (MSC) greatly limits its therapeutic benefit. Glycolysis, an energy reserve that can be quickly mobilized to meet a sudden need for energy, has not been explored as a potential contributor in the metabolic adaptation of MSC under the hostile environment. This study investigated the effect of inducible glycolgen storage (by hypoxic preconditioning) in MSC therapy for ischemia. Hypoxic preconditioning aroused glycolgen accumulation in MSC, and this glycolgen prestorage MSCs displayed better retention and limb recovery/regeneration in ischemic limb, the effect of which may be mitigated by inhibition of glycolgen storage. In addition, hypoxic preconditioning promoted prestorage glycolgen catabolism and ATP production in the subsequent long-term ischemia. We found that phosphoinositide 3-kinase/AKT/hypoxia-inducible factor 1α/glycolyn synthase kinase-3β pathways involved in hypoxic preconditioning–regulated glycolgen metabolisms. Our data indicated a vital role for glycolgen storage on MSC metabolic adaptation to ischemia, suggesting that inducible metabolic adaptation of stem cells may be a novel strategy to embellish stem cell therapy under ischemia.
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Arterioscler Thromb Vasc Biol. 2014;34:870-876; originally published online February 20, 2014;
doi: 10.1161/ATVBAHA.114.303194
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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http://atvb.ahajournals.org/content/34/4/870

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Supplemental Figures

Supplement figure I:

siRNA and adenovirus efficacy

* P<0.01 vs Control group (Con);

Supplement figure II:

Linear correlation of cell quantities with GFP signal

Colored scale bars represent optical radiance intensity in photons/second/mm²/steridian × 10^{12}.
After normoxia or hypoxic treatment, cells were subjected to in vitro ischemic model (1%O₂, in cell culture deprived of serum and glucose). The time course responses were shown. *, P<0.01 vs Con-MSC;
Supplement figure IV:

Effect of glycogenesis gene silencing on glycogenesis

A, Glucose transporter 1 (Glut 1). B, Hexokinase (HK). C, Phosphoglucomutase 1 (PGM1). *, P<0.01 vs Con-MSC;
Materials and methods

Cell culture and small interference RNA (siRNA) transfection

Mouse MSCs purchased from Cyagen Biosciences and were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 5% FBS and 50 units/ml penicillin, 50 μg/ml streptomycin. For gene silencing, the cells were grown to 70% confluence and then transfected with 25 nM (final concentration) of HIF1α siRNA (sc-35562, Santa Cruz), or glycogen synthase 1 siRNA(sc-61105, Santa Cruz) or non-targeting control siRNA (sc-37007, Santa Cruz) using Lipofectamine® 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen). After 24 h incubation at normoxia, the transfection medium was replaced with complete medium and then the cells were ready to HP.

Hypoxic preconditioning (HP), in vitro ischemic model and experimental grouping

For HP, the cells were exposed to hypoxia (1% O₂ at 37°C.) in three gas incubator (Thermo Fisher Scientific) for 24 hours. Normoxic cells were maintained at 37 °C in a 5% CO₂ and 95% air incubator. In vitro ischemia model was performed by exposing cells to hypoxia (1% O₂ at 37°C.) in cell culture deprived of serum and glucose. The grouping of cellular study is shown in Table 1.
PAS staining and glycogen determination assay
The PAS staining was conducted as previous described \(^1\). Coverslips were placed into 6-well plate, and then MSCs were plated. After incubation and HP, medium was removed and the cells were fixed with Carnoy’s fixative for 1 h. The cells were rinsed with absolute alcohol and 66% alcohol once for 2 min, followed by rinsing with deionized water for 90 s \((3\times 30\text{ s})\). The cells were treated with periodic acid solution for 10 min and then rinsed with deionized water for 90 s \((3\times 30\text{ s})\). Then the cells were treated with Shiff reagent for 15-20 min followed by 5 min with running tap water. For visualising nuclei, the cells were counterstained using Mayer’s hematoxylin for 1 min followed by running tap water for 5 min. After air-drying, the stained cells were covered with a glass slides using pure glycerol as mounting medium. For quantitative analysis, glycogen was extracted as previous described \(^2\) and the amount of glycogen was assessed by glycogen assay kit (Bioassay system).

Cell viability assay
Cell viability assay was performed by live/dead assay kit following published protocol. In detail, Cells were preconditioned in hypoxia prior to glucose removal (HP-MSCs). Cells cultured in normoxic
conditions were used as a control (Con-MSCs). Withdrawal of glucose and serum was done after washing cells twice with PBS by adding DMEM without glucose (11966-025, Gibco). After 24 hours in hypoxia, the rate of mortality was assessed by live/dead staining following the manufacturer’s protocols and imaged by fluorescence microscope (Leica DMI4000B).

**GSK-3 beta functional assay**

GSK-3 beta activity was determined by GSK-3 activity assay kit (CS0990, Sigma) following manufacture’s protocol. The assay is based on immunoprecipitation of GSK-3b using a specific anti-GSK-3b antibody. The immunoprecipitated kinase is incubated with γ-^{32}P-ATP and the incorporation of ^{32}P into the substrate is measured. The total expression of GSK3 beta and phosphorylation at Ser9 were analyzed by GSK3 beta Total + pSer9 Human In-Cell ELISA Kit (ab129731, Abcam).

**Metabolic enzyme activity and ATP content assay**

The glycogen phosphorylase (GP) activity was assayed using reported testing buffer including 50mM sodium glycerolphosphate (pH7.1), 10mM potassium phosphate, 5mM MgCl₂, 0.5mM NAD⁺, 1mM DTT, 1.6 unit phosphoglcomutase, 1.6 unit
glucose-6-phosphate dehydrogenase, and 0.2% glycogen in a total volume of 0.3ml. Reaction was started by adding homogenate into the volume and assayed at 25°C. The reaction was monitored by measuring the increase of fluorescence (Ex. 350nm, Em.470nm, HITACHI F-4600 fluorescence spectrophotometer) for NADH generation. GP catalytic activity of each group were determined and normalized to protein levels. Activity of GP under no treatment condition was set as 100%. The activities of glycolysis key enzymes, including hexokinase, phosphofructokinase and pyruvate Kinase, were test by commercially colorimetric assay kit. Hexokinase activity was assayed by Hexokinase Assay Kit (ab136957, Abcam). Briefly, glucose is converted to glucose-6-phosphate by hexokinase; the glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to form NADH, which reduces a colorless probe to a colored product with strong absorbance at 450 nm. Phosphofructokinase (PFK) Activity Colorimetric Assay Kit (MAK093, Sigma) was used to analyze PFK activity. PFK activity is determined by a coupled enzyme assay, in which fructose-6-phosphate and ATP is converted to fructose-1,6-diphosphate and ADP by PFK. The ADP is converted by the enzyme mix to AMP and NADH. The resulting NADH reduces a colorless probe resulting in a colorimetric (450 nm) product proportional to the PFK activity present. In pyruvate kinase
(PK) assay (PK Assay Kit, ab83432, Abcam), PEP and ADP were catalyzed by PK to generate pyruvate and ATP. The generated pyruvate is oxidized by pyruvate oxidase to produce color (at λ = 570 nm) and fluorescence (at Ex/Em = 535/587 nm). Since the increase in color or fluorescence intensity is proportional to the increase in pyruvate amount, the PK activity can be accurately measured.

Intra-cellular ATP content was quantified using the ATP assay kit (ab83355, Abcam), according to the manufacturer's instructions. The ATP content was expressed in fold increases over the data obtained at experimental beginning.

Mouse hindlimb ischemia model and cell transplantation

Hindlimb ischemia was induced in the 7-8 weeks-old male wild-type C57/BL mice (n=12 for each group) as previously described. Briefly, mice were anaesthetized with a single intraperitoneal ketamine (80 μg/g body weight) injection. The femoral artery was separated from the femoral nerve and vein, ligated, and excised. One day after the surgery, MSCs (10⁶ cells in 100 μl PBS) or equal volume saline were injected intramuscularly into the thigh muscle of the ischemic hindlimb. The mouse with sham operation named as sham group; mouse with hindlimb ischemia grouped as hindlimb ischemia (HI) group; mouse with both limb ischemia and MSCs treatment named as its MSC
In vivo cell tracking and morphological assessment of limb necrosis

2 weeks after cell delivery, mice were anesthetized and fluorescence imaging was performed using Carestream In-Vivo MS Fx Pro (Carestream Health, USA). After then, limb ischemia was visually evaluated. The following three grades were used to measure the degree of limb necrosis: grade 1, necrosis limited to the toes; grade 2, necrosis extending to the dorsum pedis; grade 3, necrosis extending to the crus.

Laser Doppler perfusion imaging

After in vivo fluorescence imaging and morphological assessment of necrosis, limb perfusion was assessed by laser Doppler perfusion imaging (PeriScan PIM 3 system, Perimed, Sweden). Before assay, anesthetized mice were placed on a heating pad at 37°C for 5 minutes to minimize body temperature variations. Perfusion ratio of ischemic limb versus nonischemic limb was quantified by averaging relative units of flux from the knee to the toe using PIMsoft Software (Perimed med, Sweden).

Immunofluorescence and Immunohistochemistry
For capillary density of histological assay, thigh muscle from groups were embedded in OCT, snap frozen in liquid nitrogen, and 10 μm cryosection were cut at -22°C. Capillary density was evaluated by immunofluorescence staining for CD31 (#557355, 1:200, BD) and skeletal muscle actin (ab52218, 1:200, Abcam). For cellular immunofluorescence staining, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min and incubated with blocking solution (Beyotime) for 1h at 37°C. The following primary antibodies were used: anti-Glut1 antibody (ab652, 1:200, Abcam), anti-HK 1 antibody (ab65069, 1:200, Abcam), anti-PGM 1 antibody (sc-50656, 1:200, Santa Cruz) and anti-GS 1 antibody (ab40810, 1:300, Abcam). Fluorescence-conjugated secondary antibodies (Dylight 488 and 649, 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used. The sections were counterstained with DAPI and examined with fluorescence confocal microscope (Nikon TE2000, Tokyo, Japan).

To test the MSC retention in ischemic tissue, paraffin embedded sections were stained with rabbit anti-GFP primary antibody (#2956, 1:300, Cell Signaling Tech) and goat anti-rabbit HRP-labeling secondary antibody (ab6721, 1:300, Abcam). GFP positive signal was detected by DAB substrate system (ab94665, Abcam). The GFP signal conjunct with cell nuclei was counted according to a previously
described protocol\textsuperscript{4}.

**RNA isolation and Real-Time PCR analysis**

After relative treatments, cells were dissolved in TRIZOL reagent (Invitrogen) and total RNA was extracted according to the manufacturer’s instructions. Total RNA was converted into cDNA using PrimeScript\textsuperscript{™} RT reagent Kit with gDNA Eraser (TaKaRa). The cDNA was used for quantitative Real-time PCR with specific gene primers sets (HK, PPM05501A; PGM1, PPM24785B; GS1, PPM03506G; β-actin, PPM02945B; QIAGEN). SYBR® Premix Ex Taq™II (TaKaRa) was used for the Real-time PCR. The relative abundance of target genes transcript was quantified using the comparative Ct method with β-actin as an internal control.

**Western blotting**

Cell lysates were subjected to SDS-PAGE and transferred to a PVDF membrane. Primary antibodies against the following proteins were used: p-AKT, AKT (1:300, Cell Signaling Tech), HIF-1α (1:300, Abcam), HK (1:300, SantaCruz), PGM1 (1:300, NovusBiologicals), GS1 and GAPDH (1:300; 1:1000, Proteintech). HRP-conjugated secondary antibodies were used. Signal was detected using ECL kit (Thermo Scientific).
Statistics
All values are the means ± SEM of the indicate number of determinations (n). Comparisons between the groups were determined by Student’s t-test (two groups) or one-way ANOVA followed by Bonferroni’s post-hoc test (more than two groups). \( P \) value<0.05 were considered to indicated statistical significance.

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